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A crucial role for profilin–actin in the intracellular motility of *Listeria monocytogenes*

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We have examined the effect of covalently crosslinked profilin–actin (PxA), which closely matches the biochemical properties of ordinary profilin–actin and interferes with actin polymerization *in vitro* and *in vivo*, on *Listeria monocytogenes* motility. PxA caused a marked reduction in bacterial motility, which was accompanied by the detachment of bacterial tails. The effect of PxA was dependent on its binding to proline-rich sequences, as shown by the inability of P_{H133S}xA, which cannot interact with such sequences, to impair *Listeria* motility. PxA did not alter the motility of a *Listeria* mutant that is unable to recruit Ena (Enabled)/VASP (vasodilator-stimulated phosphoprotein) proteins and profilin to its surface. Finally, PxA did not block the initiation of actin-tail formation, indicating that profilin–actin is only required for the elongation of actin filaments at the bacterial surface. Our findings provide further evidence that profilin–actin is important for actin-based processes, and show that it has a key function in *Listeria* motility.

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INTRODUCTION

A rapid and spatially controlled turnover of actin filaments is indispensable for a variety of biological processes, such as lamellipodia extension during cell motility, phagocytosis and the intracellular movement of some bacterial and viral pathogens, such as *Listeria monocytogenes* (Frischknecht & Way, 2001; Cossart & Bierne, 2001).

Among the proteins that have been implicated in the regulation of actin-filament dynamics is profilin, which was originally discovered as an actin-monomer-binding protein (Carlsson *et al.*, 1977). Profilin is localized to highly dynamic regions of cultured cells, such as the tips of spreading lamellipodia and focal adhesions (Theriot & Mitchison, 1993; Geese *et al.*, 2000), and also accumulates at the rear end of motile *Listeria* (Theriot *et al.*, 1994; Smith *et al.*, 1996; Geese *et al.*, 2000). The deletion of the genes encoding profilin alters the architecture of the actin cytoskeleton, severely impairs cell migration and leads, in most cases, to lethality (Balasubramanian *et al.*, 1994; Haugwitz *et al.*, 1994; Witke *et al.*, 2001; Schlüter *et al.*, 1997). In addition, profilin binds to cellular components implicated in the control of actin dynamics, such as phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2), Enabled (Ena)/VASP (vasodilator-stimulated phosphoprotein) proteins, N-WASP (Wiskott–Aldrich syndrome protein) and p140mDia (Frazier & Field, 1997; Schlüter *et al.*, 1997; Pollard *et al.*, 2000; Sechi & Wehland, 2000).

In vitro, profilin forms a 1:1 complex with actin (profilin–actin), which can participate in the elongation of actin filaments at their (+) ends (Korenbaum *et al.*, 1998; Kang *et al.*, 1999; Nyman *et al.*, 2002). However, when actin filament (+) ends are capped, profilin acts as an efficient inhibitor of actin nucleation. Moreover, the binding of profilin to actin enhances the exchange rate of ADP to ATP on actin (Goldschmidt-Clermont *et al.*, 1992), and profilin can synergize with other actin-monomer-binding proteins, such as thymosin-β4 and the actin depolymerizing factor (ADF)/cofilin, to increase actin-filament turnover (Pantaloni & Carlier, 1993; Didry *et al.*, 1998). Finally, a crosslinked profilin–actin (PxA) complex, the structure and function of which is similar to that of unmodified profilin–actin, inhibits both the nucleation and the elongation rate of actin filaments *in vitro* (Nyman *et al.*, 2002). This provides evidence that profilin–actin can bind to the (+) ends of actin filaments as a complex. This is supported by the observation that PxA alters actin-filament organization and blocks lamellipodial extension in cultured cells (Hajkova *et al.*, 2000).

To improve our understanding of the function of profilin–actin in actin-filament dynamics, we have studied the effect of PxA on *Listeria* motility. Our data provide further evidence that profilin–actin is important for actin-based processes and demonstrate that it has a key function in *Listeria* motility.

RESULTS

Profilin–actin impairs the intracellular motility of *Listeria*

To test the effect of PxA on the intracellular actin-based motility of *Listeria*, we injected PxA at a needle concentration of 5 mg ml^{-1}, which has been found to be effective when used on cultured cells (Hajkova *et al.*, 2000), into PtK2 cells infected with *Listeria*. Forty minutes after injection, cells were fixed and labelled with fluorescent phalloidin to detect host-cell actin filaments and the bacteria-associated actin tails. In uninjected PtK2 cells or cells that were treated with G-buffer, *Listeria* were associated with normal actin tails (Fig. 1A; arrowheads in Fig. 1C). Conversely, in PtK2 cells that were

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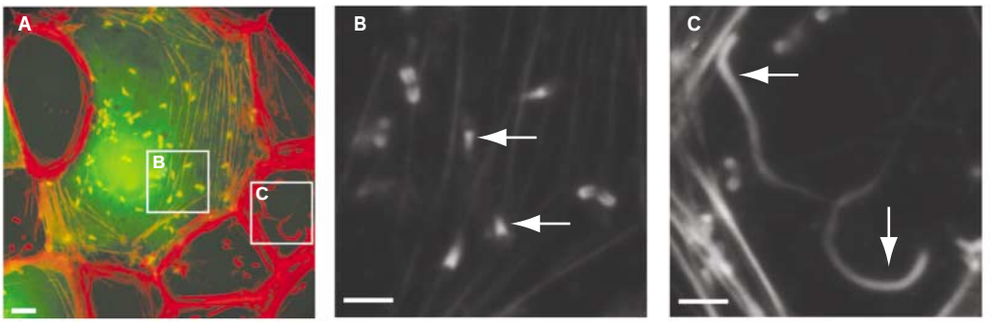

Fig. 1 | Profilin–actin impairs intracellular *Listeria monocytogenes* motility. PtK2 cells infected with *L. monocytogenes* were injected with 5 mg ml^{-1} profilin–actin (PxA; needle concentration). Oregon `Green dextran was included in the injection mixture to identify injected cells. Forty minutes after injection, PtK2 cells were fixed and stained with Texas-Red-conjugated phalloidin. The insets in (A) are enlarged in (B) and (C) In uninjected cells, *Listeria* were associated with normal actin tails (inset (C) in (A), and arrows in (C)), whereas in cells that received PxA, the bacteria were associated with short tails (inset (B) in (A), and arrows in (B)). Scale bars, 10 μm.

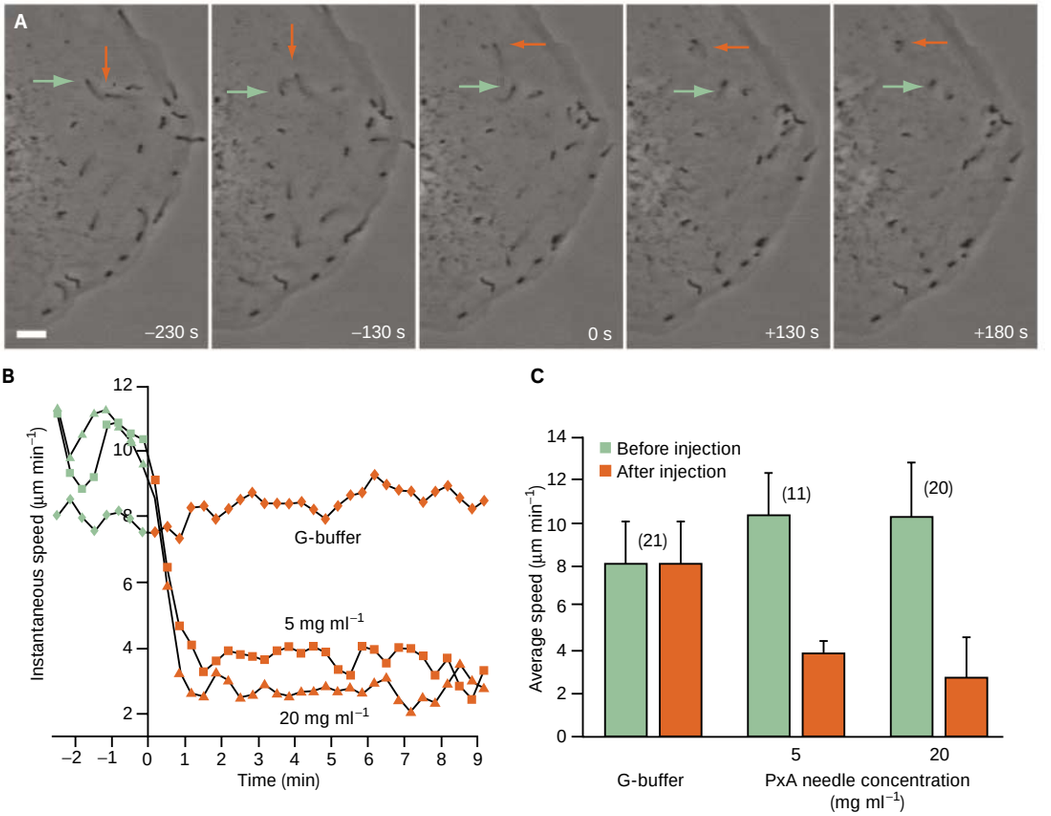

Fig. 2 | Effect of profilin–actin on *Listeria* motility. (A) Profilin–actin (PxA) reduces *Listeria monocytogenes* speed and causes actin-tail detachment. *Listeria*-infected PtK2 cells were injected with 20 mg ml^{-1} PxA and were observed by video microscopy. Before injection, *Listeria* were associated with normal actin tails and moved at an average speed of 10.2 ± 2.58 μm min^{-1} (arrows in frames for –230 s and –130 s). The injection of PxA caused an impairment of *Listeria* motility, followed by the rapid disassembly of the actin tails (arrowheads in frames for +130 s and +180 s). Three minutes after injection of PxA, *Listeria* cells were associated with a fuzzy, phase-dense material, and their movement became slow and irregular. (B,C) Effect of PxA on the instantaneous (B) and average (C) speed of *Listeria*. The injection of 5 or 20 mg ml^{-1} PxA caused a decrease in bacterial motility, whereas the injection of G-buffer had no effect. The origin of the axes corresponds to injection time in (B). Error bars in (C) represent one standard deviation above the mean. Numbers in brackets in (C) indicate the number of motile bacteria that were examined. Scale bar, 10 μm.

injected with 5 mg ml^{-1} PxA, all bacteria were associated with short actin tails (Fig. 1A; arrowheads in Fig. 1B), indicating that PxA impairs *Listeria* motility.

PxA reduces *Listeria* speed and causes tail detachment

To gain further insight into the effect of PxA on *Listeria* motility, we analysed bacterial movement by video microscopy. Before injection, *Listeria* were clearly associated with phase-dense actin tails and moved at an average speed of 10.2 ± 2.58 μm min^{-1} (Fig. 2A–C). Shortly after injection of PxA (needle concentration of 20 mg ml^{-1}), typically within 30 s, bacterial speed was reduced to an average of 2.71 ± 0.73 μm min^{-1} (Fig. 2A, green and red arrows; Fig. 2B,C; and supplementary information). Moreover, due to the impairment of actin assembly at the bacterial surface, the actin tails depolymerized within 3 min of injection of PxA (Fig. 2A, frames for 130 s and 180 s), the bacteria remained associated with a fuzzy phase-dense material (Fig. 2A, arrows in frame for 180 s), and their movement became irregular. The same result was obtained after injection of PxA at concentrations as low as 3.5 mg ml^{-1}, whereas no perturbation of bacterial motility was observed after injection of G-buffer (Fig. 2B,C; and data not shown).

Next, we analysed the effect of PxA on *Listeria* motility using MDCK (Madin–Darby canine kidney) cells that stably express green fluorescent protein (GFP)–actin. This actin derivative was incorporated into actin filaments and *Listeria* actin tails without markedly altering actin dynamics (Choidas *et al.*, 1998), and is therefore a reliable probe for analysing PxA-induced changes in actin tails in detail. Before and shortly after injection of PxA (5 mg ml^{-1}), *Listeria* cells were associated with normal actin tails (Fig. 3, frame for 10 s; compare with Fig. 1C). Approximately 30 s after injection, a break appeared between the bacterium and its tail (Fig. 3, frame for 32 s) and this, in agreement with the results obtained previously, was followed by the rapid depolymerization of the actin tail (Fig. 3, frames for 76 s and 120 s; and supplementary information). At this stage, the bacterium, which was no longer associated with its tail, was surrounded by a cloud of actin, which presumably corresponded to the fuzzy phase-dense material observed around the bacteria in phase-contrast images (Fig. 2A).

Overall, the effect of PxA on *Listeria* motility appears to be characterized by two steps: the impairment of actin-tail assembly at the bacterial surface, and the detachment of the bacteria from their actin tails.

PxA binds proline-rich regions to inhibit *Listeria* motility

Previous *in vitro* data have shown that PxA binds to the free (+) ends of actin filaments, thereby inhibiting their elongation (Hajkova *et al.*, 2000; Nyman *et al.*, 2002). Thus, it is possible that the impairment of *Listeria* motility is due to the binding of PxA to the (+) ends of the actin filaments that abut the bacterial surface. As the targeting of profilin, and possibly profilin–actin complexes, to the bacterial surface is mediated by the interaction with the central proline-rich region of Ena/VASP proteins (Geese *et al.*, 2000, 2002), the localization of which at this site is not affected by PxA (see supplementary information), it is possible that this interaction is required for the inhibitory effect of PxA on bacterial motility.

To test this hypothesis, we generated a PxA complex using the profilin mutant protein, P_{H133S}, which does not bind to poly-L-proline, although it retains the ability to associate with both monomeric actin and PtdIns(4,5)P_2 (Björkegren-Sjögren *et al.*, 1997). Injection of the complex of P_{H133S} and actin (P_{H133S}xA) at 5 mg ml^{-1} into *Listeria*-infected PtK2 cells did not change the motility of the bacteria (Fig. 4A–C; and supplementary information), indicating that poly-L-proline-dependent interactions are essential for targeting PxA to the *Listeria* surface, where it exerts its inhibitory effect. This was also supported by the observation that PxA does not affect the motility of the *Listeria* mutant strain ActA5, which expresses a modified version of ActA that lacks the central proline-rich region. As a consequence, these bacteria are unable to recruit Ena/VASP proteins and profilin, and move at a reduced speed (Smith *et al.,* 1996; Niebuhr *et al.,* 1997; Geese *et al.*, 2000; see supplementary information).

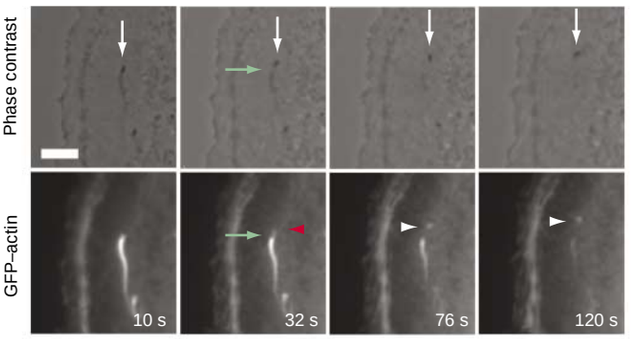

Fig. 3 | Effects of profilin–actin on *Listeria monocytogenes* motility analysed in Madin–Darby canine kidney cells expressing green fluorescent protein–actin. MDCK cells that stably expressed green fluorescent protein (GFP)–actin were infected with *Listeria* and injected with 5 mg ml^{-1} profilin–actin (PxA). Upper panels show phase-contrast images; lower panels show corresponding GFP –actin fluorescence. Shortly after injection, a break (green arrow; frame for 32 s) appeared between the bacterium (red arrowhead, lower frame for 32 s) and its tail, followed by the complete depolymerization of the actin tail (frames for 76 s and 120 s). The bacterium remained associated with a cloud of actin filaments (white arrows and arrowheads, frames for 76 s and 120 s) and moved slowly. Scale bar, 5 μm.

PxA does not inhibit formation of *Listeria* actin tails

Next, we analysed whether PxA affects the initiation of actin-tail formation. Because the rapid and severe effects of PxA on cells (Hajkova *et al*., 2000) prevented us from carrying out this investigation in living cells, we decided to use a cell-free system based on mouse cytosolic brain extracts that are able to support *Listeria* motility. This system has been shown to be useful for studying inhibitors of actin cytoskeleton components (May *et al.*, 1999). We incubated mouse cytosolic extracts with various amounts of PxA on ice for 30 min, and then added *Listeria* to induce the formation of actin tails (incubation with *Listeria* was carried out at 20 °C for 15–30 min). In control extracts, *Listeria* induced the formation of actin tails and moved at an average speed of 0.5 ± 0.16 μm min^{-1} (Fig. 5). Interestingly, in the presence of PxA, *Listeria* were still able to induce the formation of actin tails, but their motility was impaired by PxA in a concentration-dependent manner (Fig. 5; and supplementary information). This effect was not due to the dilution of the cytosolic extract (its total protein concentration was reduced by a half at the highest PxA concentration tested), as indicated by the observation that an extract diluted with G-buffer supported *Listeria* motility to the same extent as control extracts (Fig. 5).

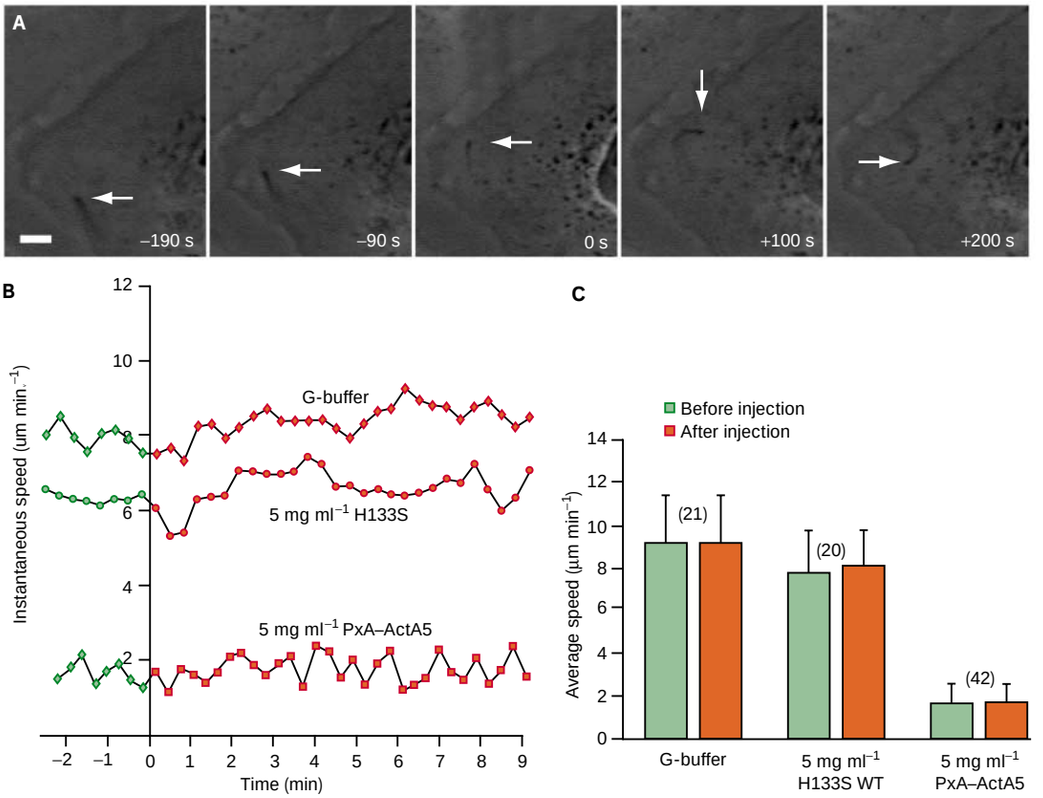

Fig. 4 | (A) The ability of profilin–actin to interact with proline-rich regions is essential for its inhibitory effect on *Listeria monocytogenes* motility. PtK2 cells infected with wild-type *Listeria* were injected with 5 mg ml^{-1} P_{H133S}xA (a complex of the H133S mutant form of profilin with actin) and observed using video microscopy. Before injection, wild-type *Listeria* were associated with actin tails and moved at normal speed (arrow in frames for –190 s and –90 s), which was not affected by the injection of P_{H133S}xA (arrow in frames for +100 s and +200 s). (B,C) Quantification of *Listeria* speed after injection with profilin–actin (PxA) or P_{H133S}xA. The effects of PxA and P_{H133S}xA on the instantaneous (B) and average (C) speed of *Listeria* ActA5 and wild-type, respectively, are shown. The injection of 5 mg ml^{-1} P_{H133S}xA or of G-buffer did not alter the motility of wild-type *Listeria*. Similarly, PxA did not change the motility of *Listeria* ActA5. The origin of the *x* axis in (B) corresponds to the time of injection. Error bars in (C) represent one standard deviation above the mean. Numbers in brackets in (C) indicate the number of motile bacteria examined.

DISCUSSION

We have shown that profilin–actin has a crucial role in actin-based *Listeria* motility. The Ena/VASP-dependent recruitment of profilin or profilin–actin at the *Listeria* surface is thought to mediate the efficient elongation of actin filaments at this site. This notion is supported by two lines of evidence. First, displacement or absence of Ena/VASP proteins from the *Listeria* surface results in a lack of profilin recruitment to this site and impairs bacterial motility (Smith *et al.*, 1996; Kang *et al.,* 1997; Niebuhr *et al.*, 1997; Geese *et al.*, 2000, 2002). Similarly, Ena/VASP mutants lacking the central proline-rich region cannot target profilin or profilin–actin to the bacterial surface, resulting in decreased and irregular *Listeria* motility (Geese *et al.*, 2002). Second, the depletion of profilin from cell extracts reduces *Listeria* motility (Marchand *et al.*, 1995), whereas its addition to a set of purified proteins that can support bacterial motility causes an increase in motility (Loisel *et al.*, 1999). Consistent with these findings, our data indicate that profilin–actin is the source of actin monomers required for efficient filament elongation at the *Listeria* surface.

Because PxA does not affect the formation of *Listeria* tails, a process that requires the actin-nucleating activity of the Arp2/3 complex (May *et al.*, 1999; Pistor *et al.*, 2000; Skoble *et al.*, 2000, 2001), we conclude that the initiation of actin tails does not depend on profilin–actin. This is also supported by the observation that PxA has no effect on the motility of *Listeria* ActA5, the movement of which depends exclusively on the nucleation and inefficient elongation of actin filaments induced by the Arp2/3 complex. Our theory is also consistent with studies showing that *Listeria* ActA provides the actin monomers necessary for the actin-nucleating activity of the Arp2/3 complex (Skoble *et al.*, 2000, 2001).

Current models for actin-filament elongation predict that profilin–actin interacts with free actin-filament (+) ends. Suggestions as to how profilin–actin might participate in actin polymerization have come from studies of the molecular organization of crystals of profilin–actin (Schutt *et al.*, 1993). Actin monomers are tightly associated in a ribbon-like arrangement, which is suggested to be an intermediate state during actin polymerization (Cedergren-Zeppezauer *et al.*, 1994). In this model, a ribbon-to-helix transformation would take

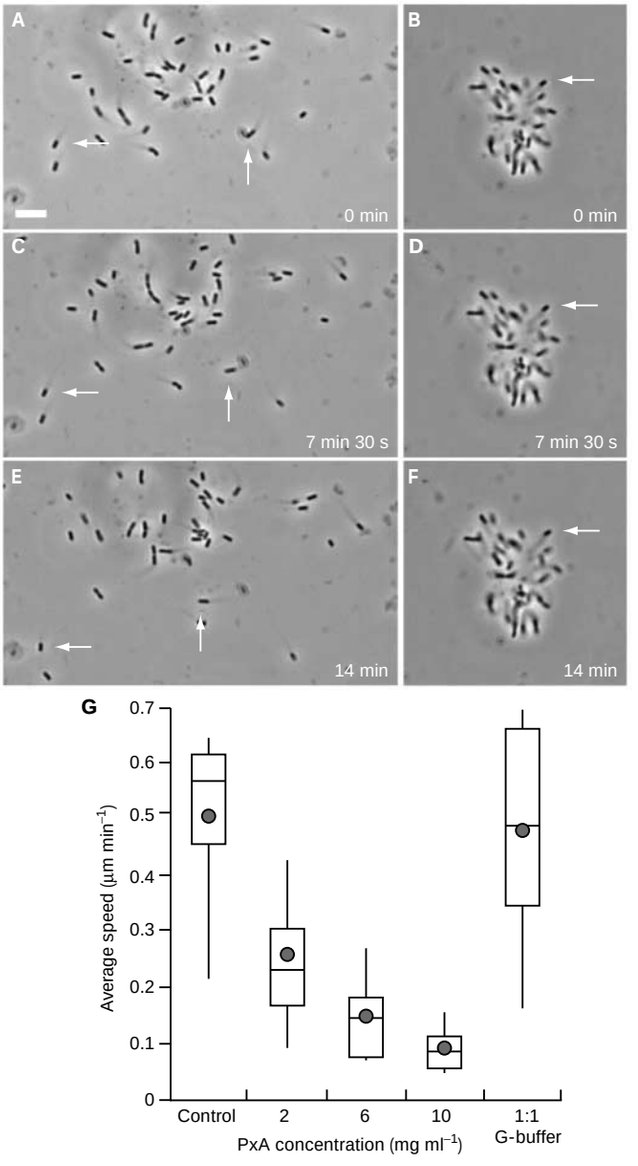

Fig. 5 | Profilin–actin does not inhibit the initiation of *Listeria monocytogenes* actin tails in mouse cytosolic brain extracts. Mouse cytosolic brain extracts were incubated with various concentrations of profilin–actin (PxA) on ice for 30 min. Bacteria were then added to the mixture, which was incubated for 15 min at 20 °C. In control extracts, *Listeria* induced the formation of actin tails and moved at an average speed of 0.5 ± 0.16 μm min^{-1} (arrows in (A,C,E); (G)). PxA induced a concentration-dependent reduction of bacterial motility (arrows in (B,D,F); (G)), but did not affect the initiation of actin tails. Note that bacteria occasionally aggregated during centrifugation before incubation with the extract. This led to movement diverging from a central point, which is clearly visible in (B,D,F). Scale bar, 10 μm. (G) Box and whiskers plots of bacterial speed. Filled circles indicate the mean; lines in the middle of the boxes indicate the median; the tops of the boxes indicate the 75th quartile and the bottoms of the boxes indicate the 25th quartile; 'whiskers' indicates the 10th and 90th percentiles, respectively.

place after the release of profilin, allowing the formation of more actin–actin contacts (Cedergren-Zeppezauer *et al.*, 1994). This is supported by the fact that PxA inhibits the incorporation of actin monomers into filaments in the presence of unmodified profilin–actin, without becoming incorporated into filaments itself (Nyman *et al.*, 2002). Thus, in the initial interaction state, PxA binds weakly to actin filament (+) ends, and the incoming actin must be freed from profilin to allow it to enter into the more stable helix conformation. The more dramatic effect of PxA on actin filament formation *in vivo* suggests that PxA blocks the actin-polymerizing machinery by binding to one of its components with high affinity. Our observations strongly suggest that the influence of PxA on *Listeria* motility is due to its high-affinity interaction with Ena/VASP proteins, and that the failure to dislodge profilin prohibits stable incorporation of actin into the growing filaments, resulting in filament breakage. Recently, Dickinson & Purich (2002) proposed a model in which *Listeria* motility depends on a 'molecular clamp' that controls actin assembly at the interface between bacteria and actin tails. We argue that PxA blocks the 'molecular clamp' and causes actin-tail detachment, as described above. It should be noted that our results do not discriminate between an actin helix formed by a ribbon-to-helix process, as described above, and the Holmes model of F-actin (Holmes *et al.*, 1990); this requires the determination of the atomic structure of F-actin.

In addition to being crucial for *Listeria* motility, profilin is required for the formin-dependent assembly of actin cables in budding yeasts, and enhances formin-induced actin filament assembly *in vitro* (Evangelista *et al.*, 2002; Sagot *et al.*, 2002). Moreover, the interaction of profilin with both actin and poly-L-proline is required for the formation of microspikes in fibroblasts (Suetsugu *et al.*, 1998) and actin cables in *Saccharomyces cerevisiae* (Sagot *et al.*, 2002), and for the intracellular motility of *Shigella flexneri* (Mimuro *et al.*, 2000; Lommel *et al.*, 2001); it is also essential for the viability of fission yeast (Lu & Pollard, 2001). These findings, together with the present study and the work of Hajkova *et al.* (2000), indicate that profilin–actin provides a pool of monomeric actin that is ready to support actin filament elongation on interaction with key regulators of actin cytoskeleton dynamics, such as WASP and N-WASP proteins, formins and Ena/VASP proteins.

METHODS

Preparation of crosslinked profilin–β-actin. Bovine profilin I and β- and γ-actin were purified as described in Hajkova *et al.* (2000) and Nyman *et al.* (2002). Non-dissociable PxA was prepared as described by Nyman *et al.* (2002). To generate P_{H133S}xA, human H133S profilin (Korenbaum *et al.*, 1998; and references therein) was crosslinked to β-actin as for wild-type profilin, except that the coupling was done at a molar ratio of 12:1 of H133S profilin to β-actin. The crosslinked profilin–β-actin was concentrated using the Ultrafree-15 Centrifugal Filter Device (Millipore), stored on ice, and used within 2–6 days.

Bacterial and cell culture, transfection and infection. The *L. monocytogenes* mutant ActA5 (Niebuhr *et al.*, 1997) and wild-type strains were grown in brain–heart infusion broth (BHI; DIFCO Laboratories) as described previously (Geese *et al.*, 2002). PtK2 and MDCK cells were grown as described in Geese *et al.* (2000). MDCK cells were transfected with GFP–actin (provided by B. Imhof) using FuGene 6, in accordance with the manufacturer's instructions. One day after transfection, positive cells were selected using 1 mg ml^{-1} G418. Infection of cells with *L. monocytogenes* was carried out as described in Geese *et al.* (2002).

Microinjection of *Listeria*-infected cells and *in vitro* motility assays. Injection of PxA was usually performed 3 h after the start of the infection, using an IM300 microinjector and an oil-driven manipulator (Narishige). We estimated that we injected a volume of PxA that was, on average, one-tenth of the cellular volume. The intracellular concentration of PxA would, therefore, be in the range of 0.5–2.0 mg ml^{-1}, depending on the experiment. Images of injected cells were acquired with an Axiovert 135 TV microscope (Zeiss) equipped with a LD-Achroplan 40×/0.60 NA (numerical aperture) lens using a C3077 CCD camera driven by the Argus 20 image processor (Hamamatsu). Images were recorded on videotape and digitalized using the Scion Frame Grabber VG-5 and Scion Image 1.62 software. The effect of PxA on the motility of *Listeria* in mouse cytosolic brain extracts was analysed as described by May *et al.* (1999). Images were acquired as described above.

Immunofluorescence microscopy and analysis of bacterial speed. Fixation and labelling of *Listeria*-infected cells and the analysis of bacterial movement were carried out as described in Geese *et al.* (2002).

Supplementary information is available at *EMBO reports* online (www.emboreports.org).

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